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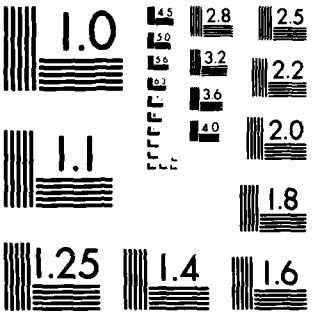

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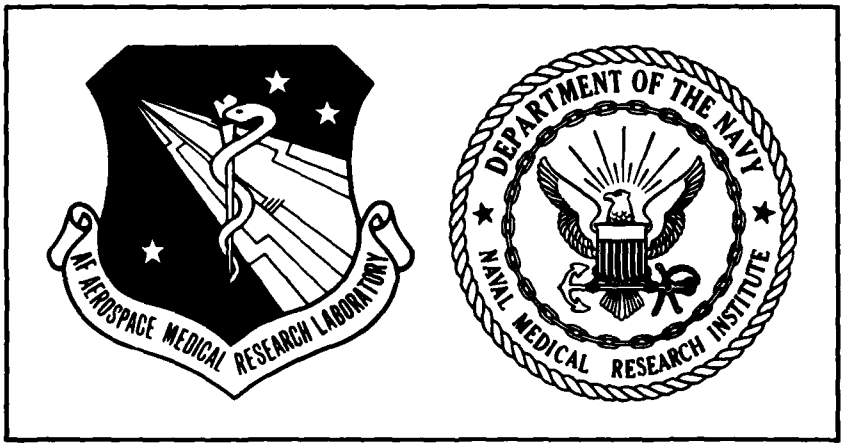
EVALUATION OF ANTIMONY THIOANTIMONATE IN THREE IN VITRO SHORT-TERM ASSAYS

ARTHUR D. LITTLE, INCORPORATED
Acorn Park, Cambridge, MA 02140

December 1984



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AEROSPACE MEDICAL DIVISION
AIR FORCE SYSTEMS COMMAND
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TECHNICAL REVIEW AND APPROVAL

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This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

BRUCE O. STUART, PhD
Director Toxic Hazards Division
Air Force Aerospace Medical Research Laboratory

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A metal compound, antimony thioantimonate, was evaluated in three mammalian cell in vitro short-term assays to determine its potential biological activity. The assays conducted were the Chinese hamster ovary (CHO) cell gene mutation assay, in the absence and presence of an Aroclor 1254-induced rat liver S9 fraction, the CHO/chromosome aberrations assay in the absence and presence of an Aroclor 1254-induced rat liver S9 fraction, and the BALB/c-3T3 neoplastic transformation assay. Under the conditions of the assays, antimony thioantimonate produced a clastogenic effect in the CHO/chromosome aberrations assay. The clastogenic response was enhanced in the presence of an exogenous metabolic activation system. The test compound produced no mutagenic activity in the CHO cells with or without an exogenous metabolic activation supplement and it produced no transformation activity in the BALB/c-3T3 neoplastic transformation assay.

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PREFACE

This is the final report of work performed by Arthur D. Little, Inc., for the United States Air Force under Work Order 0007, Contract F33615-81-D-0508, Work Unit 63020155, "Mutagenic, Teratogenic, and Carcinogenic Potential of Air Force Chemicals." This work was supported in part by the Naval Medical Research and Development Command, Research Task No. MF58524001.0006. This report describes accomplishments from July 15, 1984, to October 15, 1984. Andrew Sivak, Ph.D., was Program Manager for the program. Alice S. Tu, Ph.D., was Task Manager for this Work Order. Key personnel involved with this project included: Patricia A. Breen, B.S., Wendy C. Hallowell, B.S., Susan Evans, B.S., Karen Hatch, B.S., and Stacie L. Pallotta, B.S. Marilyn George, Biochemical Toxicology Branch, Toxic Hazards Division, Aerospace Medical Research Laboratory, was technical monitor for the Air Force.

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SUMMARY

A metal compound, antimony thioantimonate, was evaluated in three mammalian cell *in vitro* short-term assays to determine its potential biological activity. The assays conducted were the Chinese hamster ovary (CHO) cell gene mutation assay, in the absence and presence of an Aroclor 1254-induced rat liver S9 fraction, the CHO/chromosome aberrations assay in the absence and presence of an Aroclor 1254-induced rat liver S9 fraction, and the BALB/c-3T3 neoplastic transformation assay.

Under the conditions of the assays, antimony thioantimonate produced a clastogenic effect in the CHO/chromosome aberrations assay. The clastogenic response was enhanced in the presence of an exogenous metabolic activation system. The test compound produced no mutagenic activity in the CHO cells with or without an exogenous metabolic activation supplement and it produced no transformation activity in the BALB/c-3T3 neoplastic transformation assay.

LIST OF ABBREVIATIONS

CHO	Chinese hamster ovary
HGPRT	hypoxanthine-guanine phosphosphoribosyltransferase
S9	9000 xg supernatant
EMS	ethylmethanesulfonate
FCS	fetal calf serum
DFCS	dialyzed fraction of fetal calf serum
EDTA	disodium ethylenediamine-tetraacetate
PBS	phosphate-buffered saline
DMSO	dimethylsulfoxide
KCl	potassium chloride
DMN	dimethylnitrosamine
MCA	3-methylcholanthrene
CA	chromosome aberrations
$Sb(SbS_4)$	antimony thioantimonate
TG	thioguanine
CPP	cyclophosphamide
NADH	β-nicotinamide adenine dinucleotide, reduced form
NADP	β-nicotinamide adenine dinucleotide phosphate
NADPH	β-nicotinamide adenine dinucleotide phosphate, reduced form

OBJECTIVE

The objective of this study is to determine the potential biological activity of antimony thioantimonate, $Sb(SbS_4)$, a compound of principal interest to the United States Navy. Three mammalian cell in vitro short-term assays, the Chinese hamster ovary (CHO)/HGPRT gene mutation assay, the CHO/chromosome aberrations assay, and the BALB/c-3T3 neoplastic transformation assay, were utilized for this purpose.

The CHO/HGPRT assay measures the ability of a test compound to induce forward mutations at the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT) locus of Chinese hamster ovary cells on the basis that presumptive mutants defective in the enzyme HGPRT are unable to convert purine analogues such as 6-thioguanine to toxic metabolites. Hence, in a selection medium containing 6-thioguanine, the mutant cells will be able to grow, while the wild type cells are killed. To enhance detection of compounds which require metabolic activation to be mutagenic, the assay was conducted in the absence and presence of an Aroclor 1254-induced rat liver microsomal (S9) fraction.

Detection of chromosome aberrations (CA) is a classical method of assessing the effect of physical and chemical agents on the genetic apparatus of cells. Alterations in chromosomes of CHO cells as a result of exposure to a test agent are visualized in stained cytological preparations of metaphase chromosomes and are categorized as chromosome aberrations, chromatid aberrations, or numerical aberrations. The CHO/CA assay is generally carried out in the absence and presence of an Aroclor 1254-induced rat liver S9 fraction.

The BALB/c-3T3 neoplastic transformation assay is designed to measure the ability of chemical agents to induce alterations in a population of cells (derived from mouse embryo fibroblasts) from a pattern of controlled monolayer growth to one exhibiting foci of disoriented, piled up growth against the background monolayer cells. In vitro transformation is based on morphological events that mimic oncogenesis in vivo. Transformed cell populations in general assume other properties of tumor cells such as growth in semi-solid medium, and often, tumorigenicity in syngeneic, immunosuppressed host animals.

MATERIALS AND METHODS

TEST COMPOUNDS

The test compound, antimony thioantimonate, an orange powder, was provided by the U.S. Navy and used as received without further analysis. The compound was stored at room temperature.

The positive control compounds, ethylmethanesulfonate (EMS) lot #AZG and dimethylnitrosamine were purchased from Eastman Kodak Company and Sigma Chemical Company, respectively. 3-Methylcholanthrene (MCA) lot #A2 was provided by the National Cancer Institute through Illinois Institute of Technology Research Institute, and cyclophosphamide was provided by the

National Cancer Institute. These control compounds were stored in a -20°C freezer or 4°C refrigerator designated for hazardous substances. All compounds were weighed and diluted immediately prior to use on the day of the experiment.

CELL CULTURES

The cells used in the mutagenesis and cytogenetic studies were subclone BH_4 of strain K_1 of the Chinese hamster ovary (CHO) cell line. The stock cultures were originally obtained from Dr. Abraham Hsie's Laboratory (Oak Ridge National Laboratory, Oak Ridge, Tennessee) in April, 1982, and stored in liquid nitrogen.

The CHO cells were maintained in Ham's F12 medium supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, and 100 µg/ml streptomycin. They were grown in monolayer culture at 37°C in an atmosphere of 5% CO_2 and 95% humidity. For subculture, the cells were detached with a 0.25% trypsin/0.02% EDTA solution. Under these culture conditions, the cells have a doubling time of approximately 12 hours and maintain a stable karyotype of 19-20 chromosomes.

The cells used in the in vitro transformation assay were BALB/c-3T3 cells from a mouse fibroblast cell line. The original cell stock of BALB/c-3T3 clone 1-13 cells were obtained from Dr. T. Kakunaga, National Cancer Institute, in January, 1977. These cells were expanded in culture and stored in liquid nitrogen in sealed ampules at 10^6 cells/ampule. Each working stock of target cells, consisting of 50-60 ampules of cells (k series), was expanded from a frozen ampule of the original stock cells from Dr. T. Kakunaga. The medium used in the assay was Eagle's minimal essential medium supplemented with 10% fetal calf serum; penicillin and streptomycin were employed at 50 units/ml and 50 µg/ml, respectively. The cells were grown in a 5% CO_2 incubator at 37°C and 95% relative humidity.

METABOLIC ACTIVATION SYSTEM

A complete activation system consisted of F12 medium buffered with 0.02M HEPES pH 7.4, $MgCl_2$(10mM), glucose-6-PO_4(5mM), 1mM each of NADP and NADH, 0.4mM NADPH, and S9 (Aroclor 1254 induced Sprague-Dawley rat liver microsomal fraction prepared according to Ames et al. and our SOP #CB/M-526). The final concentration of S9 used was 2 mg/ml and 0.4 mg/ml in the CHO/HGPRT assay and in the CHO/CA assay, respectively. The S9 mix was prepared fresh and added to the cells immediately prior to the addition of test chemical.

CHO/HGPRT GENE MUTATION ASSAY

Initial Cytotoxicity Assay

The cytotoxic effect of Sb(SbS_4) on CHO cells was determined by a clonal assay which measured the reduction in colony forming ability of CHO cells. The CHO cells were plated at 200 cells/60 mm per dish 24 hours prior to chemical treatment. After a 16 hour exposure period to the test chemical (5 hours in the presence of S9 metabolic activation), the medium containing test

chemical was removed, the cells were rinsed with phosphate buffered saline (PBS) and fresh medium added. The cells were incubated in a 5% CO_2-incubator at 37°C for 7 days to allow colonies to develop. At the end of the incubation period, the culture dishes were rinsed with PBS, fixed in 100% ethanol and stained with 5% Giemsa and the colonies were counted. The cloning efficiency and the surviving fraction were calculated from the clonal counts obtained in the control and treated conditions. The concentrations selected for the mutation assay was based on results of this clonal assay.

Parallel cytotoxicity assay

In parallel with the mutation assay, CHO cells were plated at 200 cells/60 mm dish and treated with test chemical at the same time as the mutation dishes to determine the cytotoxic effect of the test compound at the concentrations tested for the mutation assay. The clonal assay procedure was as described for the initial cytotoxicity assay.

CHO/HGPRT Mutation Assay Procedure

Mutation induction at the HGPRT locus was measured as described in our standard operating procedure #CB/M-806b. Briefly, cells were plated at $5X10^5$/100 mm dish in Ham's F12 medium containing 5% dialized fetal calf serum (DFCS). After 24 hours, duplicate cultures were treated with test chemical, the primary stock of which was prepared in dimethylsulfoxide (DMSO). The solvent control was the highest concentration of DMSO used in the experiment and the positive control was ethylmethanesulfonate (EMS) at 248 µg/ml in the absence of S9 or dimethylnitrosamine (DMN) at 100 µg/ml in the presence of S9. The cells were treated for 16 hours (5 hours in the presence of S9) after which the medium was removed, the cells were rinsed with PBS, and fresh culture medium containing 5% DFCS was added. After approximately 24 hours of incubation, the cells from each dish were trypsinized, counted and replated in duplicate at $1x10^6$ cells/100 mm dish in 10 ml of medium containing 5% DFCS. The cells were subcultured once every 2-3 days to allow a total expression period of 7-9 days. To select for thioguanine-resistant mutants, the cells were trypsinized, counted and replated at $2x10^5$ cells/100 mm dish in 10 ml of hypoxanthine-free growth medium containing 5% DFCS and 10 mM 6-thioguanine (TG). Ten dishes (5 from each duplicate for a total of $2x10^6$ cells screened) were used for each test condition. From the same stock cells, five replicates were plated with 200 cells/60 mm in medium without TG to determine cloning efficiency. The cultures were incubated for 6-9 days, the dishes were then rinsed with PBS, fixed in methanol, stained with 5% Giemsa, and the colonies counted.

Data Quantitation

The mutation frequency was calculated by dividing the total number of TG-resistant colonies scored by the number of cells plated ($2x10^6$), corrected for the cloning efficiency and is expressed as mutants per 10^6 clonable cells.

Acceptability of Assay and Criteria for Response

A mutation assay is considered acceptable if it meets the following criteria:

- the cloning efficiency of the CHO cells is $\geq$60%
- three of the four test concentrations have an acceptable number of cells ($5x10^5$) analyzed for HGPRT mutation.
- the positive control (EMS or DMN) induces a statistically significant (Student's t-test, $p<0.01$) mutation frequency above the untreated or solvent control.

A test chemical is considered positive in the mutation assay if:

- it induces a statistically significant (Student's t-test, $p<0.01$) mutation frequency above the control in a dose-dependent manner; and
- a net increase in mutant colonies of treated above the control is observed in at least two of the concentrations tested.

These criteria are not absolute and other extenuating factors may enter into the final interpretation of the results.

CHO/CHROMOSOME ABERRATIONS ASSAY

Initial Cytotoxicity Assay

The concentrations of test chemical used in the assay were determined from a cytotoxicity assay procedure which measures the reduction in cell number of CHO cells in mass culture after a 24 hour exposure period to the test chemical. Exponentially growing CHO cells were plated for 24 hours, the cells were then treated with varying concentrations of the test chemical for a treatment period of 24 hours (5 hours in the presence of S9). At the end of the treatment, the cells were trypsinized and counted. The surviving fraction was determined by calculating the ratio of the mean cell counts of each treated culture to that of the untreated control. The highest concentration of test chemical used in the assay was the concentration resulting in a relative surviving fraction of approximately 0.20-0.50.

CHO/CA Assay Procedure

Exponentially growing CHO cells were plated at $3x10^5$ cells/100 mm dish and incubated for 24 hours. Duplicate cultures were treated with test chemical for 24 hours in the absence of S9. (In the presence of S9, chemicals were removed after 5 hours and incubated for an additional 19 hours without test chemicals). Two hours prior to the collection of cells, colcemid was added to the culture at a final concentration of 10^{-5}M. At the end of the incubation period, the cells were collected by trypsinization, swelled in hypotonic medium (0.075 M KCl), and fixed in methanol:acetic acid (3:1). The fixed cells were dropped on clean slides, stained in a 4% Giemsa

solution, pH 6.8, rinsed in water, and air dried. The mitotic index for each test condition was determined by counting the number of cells in mitosis in 1000 randomly selected cells on the slide. Chromosome aberrations were scored microscopically in 100 metaphases for each test condition, 50 from each duplicate. The following categories of aberrations were scored and recorded:

Chromosome Aberrations - changes in the configurations of whole chromosomes observed at homologous sites of both chromatids. These include markers (acentrics, dicentrics, rings, translocations), breaks, fragments, and gaps.

Chromatid Aberrations - changes involving individual chromatids of a chromosome. These include interchanges (quadraradials, triradials), breaks, fragments, and gaps.

Numerical Aberrations - changes involving many or all chromosomes within a cell. These are scored as pulverized chromosomes or cells with greater than 10 aberrations.

Data Quantitation

Chromosome and chromatid gaps were scored but not included as aberrations in the quantitation of data. For each test condition, the mean number of aberrations per cell $\pm$ the standard deviation and the standard error of the mean (S.E.M.) were calculated. Also, the percentage of cells with aberrations were determined from total metaphases scored. Cells with numerical aberrations were excluded in the calculation of aberrations per cell but included in the calculation of cells with aberrations.

Acceptability of Assay and Criteria for Response

An assay is acceptable if:

- a minimum of 60 metaphases are scored for the test condition to be included in the calculation
- two of the four concentrations tested have acceptable number of metaphases scored
- the positive control (EMS or CPP) induces a statistically significant (Student's t test $p<0.01$) increase in aberrations over the control.

A test chemical is considered positive in the assay if it induced a statistically significant (Student's t test, $p<0.01$) increase in aberration frequency above the control in more than one of the concentrations tested. A dose-dependent effect is corroborative evidence of a positive response. The following guideline may also be used in the judgement of a positive response in this assay.

Response	% Cells with Aberrations
-	<4.9
±	5.0 - 9.9
+	10.0 - 19.9
++	20.0 - 49.9
+++	>50.0

BALB/c-3T3 CELL TRANSFORMATION ASSAY

Initial Cytoxicity Assay

The effect of the test chemical on the survival of BALB/c-3T3 cells was determined by a reduction of cell number in mass culture after treatment with the test chemical. Exponentially growing BALB/c-3T3 cells were plated at $5x10^5$/35 mm well of cluster dishes. After 24 hours, duplicate cultures were treated with varying concentration of test chemical for 3 days, the exposure time for the transformation assay. At the end of the treatment period, the cells were trypsinized and counted. The surviving fraction of cells treated with chemical was calculated by comparing to untreated cells which was considered 100% survival. The highest test concentration used in the transformation assay was that resulting in 10-20% cell survival.

Parallel Cytotoxicity Assay

The cytotoxicity of the test chemical at concentrations tested in the transformation was determined as follows: At the end of the three day treatment period, two plates selected from each experimental set were rinsed with PBS, the cells trypsinized and counted. An aliquot of cells from each test condition was replated at 100 cells/plate (5 plates per set) to determine the cloning efficiency of the cells.

BALB/c-3T3 Transformation Assay Procedure

Dishes for transformation assay were plated with cells expanded from frozen stock at 10^4 cells per 60 mm plate. At least 22 dishes were set up for each test condition. Twenty-four hours later, the test chemicals were added to the appropriate plates. After a three-day treatment, the medium was removed, the plates were replenished with fresh medium and incubated for a total of approximately four weeks. The medium was changed every 4-7 days during the incubation period. At the end of the incubation period, the plates were fixed with methanol and stained with 2-3% Giemsa. Each stained plate was examined for foci under a dissecting microscope. Foci are dense areas of cells over the background monolayer. Foci are classified into 3 types as described by Reznikoff et al.(Cancer Res. 33:3239, 1973). Only Type III foci (aggregation of multilayered densely stained cells that are randomly oriented and exhibiting criss-cross array at the edge of the focus) are scored.

Data Quantitation

The mean number of Type III foci per plate and the standard error of the mean as well as the fraction of plates with Type III foci for each experi-

mental set were calculated. To determine if the number of foci/plate of the treated sets is significant ($P<0.05$) above that of the control, a modified t-statistic is carried out.

$\bar{X}_C$ and $\bar{X}_T$ = mean foci/plate of the control and treated sets, respectively; SE_C and SE_T = standard error of the control and treated sets; and n_C and n_T = number acceptable control and treated plates.

$$t = \frac{\left|\bar{X}_C - \bar{X}_T\right|}{\sqrt{(SE_C)^2 + (SE_T)^2}}$$

$$DF = \frac{\left|SE_C^2 + SE_T^2\right|^2}{\frac{SE_C^4}{n_C} + \frac{SE_T^4}{n_T}}$$

Acceptability of Assay and Criteria for Response

An assay is acceptable if it meets the following criteria:

- A minimum of 10 plates must be scored for each experimental set to be included in the calculation.
- Three of the four test concentrations have the minimum number of acceptable plates.
- The upper limit of Type III foci/plate for the untreated control is 0.75. The lower limit of Type III foci/plate for the positive control (MCA, 2 µg/ml) is 1.20.

A test chemical is considered positive in the assay if it produces a statistically significant ($P<0.05$) increase in Type III foci/plate above the untreated control in at least two of the concentrations tested. The fraction of plates with foci and a positive dose-response are corroborative data used in the final judgement of a positive response.

RESULTS

SOLUBILITY OF TEST CHEMICAL

The solubility of antimony thioantimonate in phosphate buffered saline solution and in a number of organic solvents commonly used for in vitro short term assays was determined. The results in Table 1 show that $Sb(SbS_4)$ was

uniformly insoluble in acetone, ethanol, and PBS and only slightly soluble in DMSO. Even at 1 mg/ml, the lowest concentration screened, the test chemical was not completely soluble in DMSO. The solubility of the test compound improved slightly with sonication. However, when further diluted in PBS, some of the test chemical again precipitated out of solution. In the various assays conducted, $Sb(SbS_4)$ was dissolved in DMSO and added directly in a small volume (20-50 µl) to the culture medium. The final concentration of $SbSbS_4$ in the test medium that remained in solution under the conditions of the assay was not known.

CHO/HGPRT MUTATION ASSAY

The cytotoxicity of $Sb(SbS_4)$ on the CHO cells in the absence and presence of an exogenous metabolic activation system was determined by a clonal survival assay. The results are shown in Tables 2 and 3, respectively. Despite the limited solubility, $Sb(SbS_4)$ was cytotoxic on the CHO cells at concentrations higher than 10 µg/ml in the absence of the S9 fraction (Table 2). At 100 µg/ml, $Sb(SbS_4)$ was equally cytotoxic whether the stock solutions were dissolved in DMSO or in PBS, suggesting that although the test chemical was more soluble in DMSO than in PBS, the final concentration present in the test medium was not different between the two primary solvents used.

In the presence of a rat liver S9 fraction, $Sb(SbS_4)$ was less cytotoxic. At 100 µg/ml, a surviving fraction of 0.71 was observed compared to that of <0.01 in the absence of S9. The reduced toxicity, however, may not necessarily be due to the S9 fraction, since a shorter exposure period of 5 hours was used in the presence of S9 as compared to 16 hours in the absence of an exogenous activation system.

The results of the CHO/HGPRT mutation assay on $Sb(SbS_4)$ in the absence and presence of S9 are shown in Tables 4 and 5, respectively. Consistent with the initial cytotoxicity data, $Sb(SbS_4)$ produced a dose-dependent increase in cytotoxicity at the concentration range of 10-80 µg/ml (Table 4). Although $Sb(SbS_4)$ was less toxic in the presence of S9 (Table 5), it was not tested above 100 µg/ml due to the insolubility of the compound. Even at the concentrations tested, precipitates were still noted in the culture plates 2-3 days after removal of the test chemical and subsequent to two medium changes. Nevertheless, the results show that $Sb(SbS_4)$ was not mutagenic with or without an exogenous metabolic activation system under the conditions of the assay which produced a dose-dependent cytotoxic effect.

CHO/CHROMOSOME ABERRATIONS ASSAY

The cytotoxic effect of $Sb(SbS_4)$ on CHO cells under conditions of the chromosome aberrations assay was examined in a mass culture assay. Results in Table 6 show that in the absence of the S9 fraction, the number of CHO cells was reduced by more than 40% after a 24-hour exposure to 30 µg/ml of $Sb(SbS_4)$. The cytotoxicity of $Sb(SbS_4)$ was slightly reduced in the presence of the S9 fraction (Table 7). Cyclophosphamide, which is nontoxic in the absence of S9, produced a dose-dependent cytotoxic effect in the presence of S9.

Results of the cytogenetic assay show that in the absence of an exogenous metabolic activation system, $Sb(SbS_4)$ induced a significant increase in chromosome aberrations above the solvent control at 60 µg/ml, the highest concentration tested (Table 8). However, no significant chromosomal effect was observed at 30 µg/ml, although there was a reduction in mitotic index at a concentration as low as 15 µg/ml. The clastogenic effect of $Sb(SbS_4)$ was enhanced in the presence of a rat liver S9 fraction. The increase in chromosone aberrations was dose-dependent between the concentrations of 12.5 and 100 µg/ml (Table 9), although the test chemical was not completely soluble at these test concentrations.

BALB/c-3T3 CELL TRANSFORMATION ASSAY

In contrast to the CHO cells, $Sb(SbS_4)$ was highly toxic to the BALB/c-3T3 cells. The relative surviving fraction was less than 10% at a concentration as low as 1.0 µg/ml (Table 10). The substantially higher cytotoxic effect on the BALB/c cells could be due in part to the longer exposure period of 3 days in this assay compared to the maximum 1 day exposure in the CHO assays.

Despite its cytotoxic effect, $Sb(SbS_4)$ exhibited no transforming activity under the conditions of the assay (Table 11). At the concentrations tested, $Sb(SbS_4)$ produced a dose-dependent cytotoxic effect.

CONCLUSIONS

The metal compound antimony thioantimonate was highly insoluble in culture medium. Consequently, not all the chemical was in the soluble state under the assay conditions of the CHO/HGPRT assay and the CHO/CA assays. Nevertheless, a dose-dependent cytotoxic effect was observed in the CHO cells. Under the limited solubility conditions, $Sb(SbS_4)$ was not mutagenic in the CHO cell, but was highly clastogenic, especially in the presence of an exogenous metabolic activation system. The test compound did not produce a transformation response in the BALB/c-3T3 cells, although it was highly toxic to these mouse fibroblasts (Table 12).

TABLE 1

SOLUBILITY SCREENING OF ANTIMONY THIOANTIMONATE
IN VARIOUS SOLVENTS

A. Without Sonication

mg/ml	DMSO	Acetone	Ethanol	PBS
1000	Insoluble	Insoluble	Insoluble	Insoluble
100	Slightly Soluble	Insoluble	Insoluble	Insoluble
10	Slightly Soluble	Insoluble	Insoluble	Insoluble
1	Almost Soluble	Insoluble	Insoluble	Insoluble

B. With 1 Minute Sonication

mg/ml	DMSO	Acetone	Ethanol	PBS
1000	Insoluble	Insoluble	Insoluble	Insoluble
100	Slightly Soluble	Insoluble	Insoluble	Insoluble
10	Homogenous Suspension	Slightly Soluble	Slightly Soluble	Slightly Soluble
1	Not Done	Slightly Soluble	Slightly Soluble	Slightly Soluble

TABLE 2

CYTOTOXICITY OF ANTIMONY THIOANTIMONATE ON CHO CELLS
WITHOUT EXOGENOUS METABOLIC ACTIVATION - CLONAL ASSAY

Chemical	Concentration µg/ml	Colonies/Plate ±S.D.[a]	% Cloning Efficiency	Treated/ Control
Control	-	167 ± 13.1	83.5	1.00
DMSO	1%	156 ± 6.8	78.0	0.93
$Sb(SbS_4)$[b]	0.3	149 ± 16.1	74.5	0.89
	1.0	139 ± 17.4	69.5	0.83
	3.0	161 ± 12.7	80.5	0.96
	10.0	156 ± 6.8	78.0	0.93
	30.0	114 ± 10.7	57.0	0.68
	100.0	0.7 ± 0.6	0.4	<0.01
$Sb(SbS_4)$[c]	100.0	2 ± 1.5	1.0	0.01
	300.0	0	-	-
	1000.0	0	-	-

[a] Mean of triplicates ± standard deviation
[b] Primary stock of $Sb(SbS_4)$ dissolved in DMSO
[c] Primary stock of $Sb(SbS_4)$ dissolved in PBS

TABLE 3

CYTOTOXICITY OF ANTIMONY THIOANTIMONATE ON CHO CELLS WITH AROCLOR 1254-INDUCED RAT LIVER S9 ACTIVATION - CLONAL ASSAY

Chemical	Concentration µg/ml	Colonies/Plate ±S.D.[a]	% Cloning Efficiency	Treated/ Control
Control	(w/o S9)	167 ± 11.0	83.5	-
Control	(w/S9)	178 ± 7.5	89.0	1.00
DMSO	1%	169 ± 11.4	84.5	0.95
$Sb(SbS_4)$	0.1	177 ± 9.3	88.5	0.99
	0.3	177 ± 13.2	88.5	0.99
	1.0	169 ± 9.9	84.5	0.95
	3.0	153 ± 5.5	76.5	0.86
	10.0	172 ± 8.5	86.0	0.97
	30.0	162 ± 9.5	81.0	0.91
	100.0	127 ± 41.5[b]	63.5	0.71

[a] Mean of triplicates ± standard deviation.
[b] Colonies much smaller than control.

TABLE 4

CHO/HGPRT GENE MUTATION ASSAY ON ANTIMONY THIOANTIMONATE
WITHOUT EXOGENOUS METABOLIC ACTIVATION

		Cytotoxicity		Mutagenicity		
Chemical	Concentration µg/ml	% Cloning Efficiency	Treated/ Control	Total Mutants	% Cloning Efficiency	Mutation Frequency[a]
Control	0	55.0	0.89	34	67.5	25.0
DMSO	0.5%	61.5	1.00	36	137.0	13.1
$Sb(SbS_4)$	10	42.0	0.68	26	80.5	16.0
	20	39.5	0.64	9	94.5	4.7
	40	11.0	0.18	26	73.5	17.6
	60	2.0	0.03	32	95.0	16.8
	80	0.2	<0.01	0	149.0	<0.3[b]
EMS	248	45.0	0.73	679	63.5	530.5[c]

[a] Expressed as mutants per 10^6 clonable cells.
[b] Statistically below the control and solvent control.
[c] Statistically above the control and solvent control.

TABLE 5

CHO/HGPRT GENE MUTATION ASSAY ON ANTIMONY THIOANTIMONATE
WITH AROCLOR 1254-INDUCED RAT LIVER S9 METABOLIC ACTIVATION

			Cytotoxicity		Mutagenicity		
	Chemical	Concentration µg/ml	% Cloning Efficiency	Treated/ Control	Total Mutants	% Cloning Efficiency	Mutation Frequency[a]
	Control	(w/o S9)	90.0	1.00	29	92.5	15.6
	DMSO	(w/S9)	90.0	1.00	10	37.0	13.5
	DMSO	1%	86.5	0.96	3	81.0	1.9
	$Sb(SbS_4)$	20	86.0	0.96	9	77.5	5.8
		40	78.5	0.87	15	73.0	10.3
		60	86.0	0.96	14	54.0	13.0
		80	83.0	0.92	13	58.5	11.0
		100	79.0	0.88	20	57.5	17.2
	DMN	100	82.5	0.92	54	76.0	35.5[b]

[a] Expressed as mutants per 10^6 clonable cells.

[b] Statistically significant above the S9 and solvent control.

TABLE 6

CYTOTOXICITY OF ANTIMONY THIOANTIMONATE ON MASS CULTURE OF CHO CELLS WITHOUT EXOGENOUS METABOLIC ACTIVATION

Chemical	Concentration (µg/ml)	Cells/Plate[a]	Treated/Control
Control	-	3.2×10^6	-
$Sb(SbS_4)$	0.1	3.4×10^6	1.06
	0.3	3.1×10^6	0.97
	1.0	3.2×10^6	1.00
	3.0	2.5×10^6	0.78
	10.0	2.5×10^6	0.78
	30.0	1.8×10^6	0.56
	100.0	1.3×10^6	0.41

[a] Mean of duplicate plates.

TABLE 7

CYTOTOXICITY OF ANTIMONY THIOANTIMONATE ON MASS CULTURE OF CHO CELLS WITH AROCLOR 1254-INDUCED RAT LIVER S9 ACTIVATION

Chemical	Concentration (µg/ml)	Cells/Plate	Treated/Control
Control	(w/o S9)	12.3×10^5	-
Control	(w S9)	13.3×10^5	1.00
$Sb(SbS_4)$	1.0	12.0×10^5	0.90
	3.0	10.0×10^5	0.75
	10.0	12.7×10^5	0.96
	30.0	10.5×10^5	0.79
	100.0	8.4×10^5	0.63
CPP	10.0	9.2×10^5	0.69
	20.0	7.4×10^5	0.56
	30.0	4.9×10^5	0.37

TABLE 8

CHO/CHROMOSOME ABERRATIONS ASSAY ON ANTIMONY THIOANTIMONATE
WITHOUT EXOGENOUS METABOLIC ACTIVATION

CHEMICAL CONCENTRATION (µg/ml)	Control	DMSO 0.5%	Antimony Thioantimonate 7.5	15	30	60	EMS 248
MITOTIC INDEX (%)	6.7	6.9	6.9	5.4	5.9	4.5	4.4
CHROMOSOME ABERRATIONS							
MARKER	0	3	3	2	0	5	10
BREAK	0	1	4	2	6	8	14
FRAGMENT	0	3	1	1	1	10	4
GAP	0	2	0	0	1	2	1
CHROMATID ABERRATIONS							
INTERCHANGE	0	0	0	0	1	3	2
BREAK	1	1	0	1	0	8	12
FRAGMENT	0	1	1	0	0	1	5
GAP	1	5	4	0	2	11	13
NUMERICAL ABERRATIONS							
PULVERIZED	0	0	0	0	0	1	0
> 10 aberrations	0	0	0	0	0	0	0
TOTAL ABERRATIONS[a]	1	9	9	6	8	36	47
CELLS SCORED	100	100	100	100	100	100	100
ABERRATIONS/CELL ± S.E.M.	0.01±0.01	0.09±0.03	0.09±0.03	0.06±0.02	0.08±0.03	0.36±0.08	0.47±0.07
% CELLS WITH ABERRATIONS	1	9	8	6	8	23	35
t-TEST SCORE	-	-	0	0.80	0.25	3.33	4.85
p<0.05	-	-	no	no	no	yes	yes
p<0.01	-	-	no	no	no	yes	yes

[a] excluding gaps.

TABLE 9

CHO/CHROMOSOME ABERRATIONS ASSAY ON ANTIMONY THIOANTIMONATE
WITH AROCLOR 1254-INDUCED RAT LIVER S9 METABOLIC ACTIVATION

CHEMICAL CONCENTRATION (μg/ml)	Control w/o S9	Control w/S9	Antimony Thioantimonate 12.5	25.0	50.0	100	CPP 30
MITOTIC INDEX (%)	6.7	6.6	7.6	6.8	3.0	4.0	3.2
CHROMOSOME ABERRATIONS							
MARKER	0	0	6	13	7	10	5
BREAK	0	0	5	7	12	13	108
FRAGMENT	2	1	10	25	36	21	51
GAP	0	1	1	4	2	3	7
CHROMATID ABERRATIONS							
INTERCHANGE	0	0	1	1	0	5	24
BREAK	0	0	3	5	8	3	18
FRAGMENT	0	0	1	0	2	1	27
GAP	4	2	6	10	4	4	21
NUMERICAL ABERRATIONS							
PULVERIZED	0	0	0	0	0	0	0
> 10 aberrations	0	0	0	0	0	0	5
TOTAL ABERRATIONS[a]	2	1	26	49	65	53	238
CELLS SCORED	100	100	100	100	100	100	100
ABERRATIONS/CELL ± S.E.M.	0.02±0.01	0.01±0.01	0.26±0.07	0.49±0.09	0.65±0.11	0.53±0.07	2.45±0.19
% CELLS WITH ABERRATIONS	2	1	17	32	40	41	85
t-TEST SCORE	-	-	8.38	8.80	6.08	7.17	13.13
p<0.05	-	-	yes	yes	yes	yes	yes
p<0.01	-	-	yes	yes	yes	yes	yes

[a] excluding gaps and cells with numerical aberrations.

TABLE 10

CYTOTOXICITY OF ANTIMONY THIOANTIMONATE ON MASS CULTURE OF BALB/c-3T3 CELLS

Chemical	Concentration	Cells/Plate[a]	Treated/Control
Control	-	1.5×10^5	-
DMSO	0.5%	1.5×10^5	1.00
$Sb(SbS_4)$	1.0	1.3×10^4	0.09
	3.0	6.7×10^3	0.04
	10.0	5.0×10^3	0.03
	30.0[b]	2.8×10^2	<0.01
	100.0[b]	2.0×10^3	0.01

[a] Mean cell number of duplicate plates
[b] Chemical coated bottom of plates

TABLE 11

BALB/c-3T3 TRANSFORMATION ASSAY ON ANTIMONY THIOANTIMONATE

Chemical	Control	DMSO	Antimony Thioantimonate					MCA
Concentration (μg/ml)		0.5%	0.05	0.10	0.25	0.50	1.00	2.0
Total Foci/ Total Plates	7/20	6/19	4/20	4/20	5/20	2/20	3/19	34/20
Foci per Plate ± S.E.M.[a]	0.35±0.11	0.32±0.13	0.20±0.09	0.20±0.09	0.25±0.12	0.10±0.07	0.16±0.09	1.70±0.27
Total Plates with Foci/Total Plates	7/20	6/20	4/20	4/20	4.20	2/20	3/19	15/20
Ratio	0.35	0.30	0.20	0.20	0.20	0.10	0.16	0.75
T-Statistic	-	0.16	0.90	0.90	0.58	1.67	1.18	4.22
Degrees of Freedom	-	39	37	37	39	31	35	28
Significant Transformation Activity ($p<0.05$)	-	No	No	No	No	No	No	Yes
Cells/Plate x 10^5	6.5	6.5	4.5	4.4	2.5	1.3	0.49	1.9
Treated/Control	-	1.00	0.67	0.56	0.38	0.20	0.08	0.29
Clones/100 Cells±S.E.M.[a]	70±6.1	70±3.6	62±2.7	59±3.3	55±3.3	38±3.6	22±4.1	23±3.3
Treated/Control	-	1.00	0.89	0.84	0.79	0.54	0.31	0.33

[a] Standard error of the mean.

TABLE 12

BIOLOGICAL ACTIVITY OF ANTIMONY THIOANTIMONATE
IN THREE IN VITRO MAMMALIAN SHORT-TERM ASSAYS - SUMMARY

Assay	w/o Activation	w/ Activation
CHO/HGPRT	-	-
CHO/CA	+	++
BALB/c-3T3	-	N.D.

- = negative
+ = positive
++ = highly positive
N.D. = not done

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